JOANNE STUBBE: This is the second recitation on cholesterol, and it's really focused on this question of how do you sense cholesterol in a membrane? So that's really a tough problem.

And they've developed new tools, and that's what we're going to be talking about-- what the tools are, and whether you would think they were adequate to be able to address this question about what kinds of changes in concentration of cholesterol. Number one, can you measure them? And number two, what effects do they have, in terms of whether you're going to turn on cholesterol biosynthesis and uptake, because you need more cholesterol, or you're going to turn the whole thing off?

So we've been focusing, as we've described in the last few lectures, in the endoplasmic reticulum. And what would the cholesterol-- what kinds of changes in cholesterols did they see in the experiments they were doing in this paper? What were the range of changes that they saw?

AUDIENCE: 3% to 10%?

JOANNE STUBBE: Yeah, so see, something low. Say they were trying to do this same experiment in the plasma membrane-- how do we know it's the ER membrane that does this sensing? That's what the whole paper is focused on, that's what everything we've focused on in class.

Say you wanted to do a similar kind of experiment in the plasma membrane, do you remember what I said about the levels of cholesterol? So they distributed throughout the cell, in all membranes. Where is the most cholesterol? So if you don't remember, it's the plasma membrane.

So say, instead of having 7% or 8% of the lipids cholesterol, say you had 40%-- that's an over-exaggeration-- do you think this kind of an experiment would be hard to do, that they've talked about in this paper? So you would want to do this-- if you tried to do the same experiment with the plasma membrane?

So the key issue that you need to think about, is go back and look at the changes-- they did a whole bunch of different experiments. The numbers are squishy, but they came up with numbers that reproduced themselves, I thought, in an amazing way. But now say you wanted to do this in the plasma membrane, where the levels of cholesterol are much higher.

Do you think it would be easy to do? Using the same tech techniques that are described, that we're going to discuss, or not? And what would the issues be? Yeah?

AUDIENCE: So they had to deplete the cholesterol from the membrane, and so that would probably be hard to deplete it to a level that's low enough, so that you don't get the activity. Right?

JOANNE STUBBE: So, I don't know. So that's an interesting question. So you'd have to deplete-- so that's going to be it, we're going to have to control the cholesterol levels. But what change-- if you looked at the changes in levels of cholesterol in the ER, how much did they change?

They change from what to what? From-- 2% to 7%. Say that you were in that same range of change that was going to turn on a switch in the plasma membrane. And say you could control the levels. Do you think it would be easy to see that?

So you start with 40%, say, that's the norm. Say the change was very similar to what you see in the change in the ER-- do you think that would be easy to detect? No, because now you have two big numbers, and there's a huge amount of error in this method of analysis.

So those are the kinds of things I'm trying to get you to think about. I don't know why it's the ER-- I mean, everybody's focused on the ER. Could cholesterol and other organelles have a different regulatory mechanism? Or somehow be connected, still, to what's going on in the ER? Could be-- I mean, you start out with the simplest model you can get and you test it, but then as you learn more, or we have more and more technology, we learn new things, you go back and you revisit and rethink about what's going on.

So the key question is, it's really this switch of having cholesterol that keeps it in the membrane, or not having cholesterol. And the question is, what are the differences in the levels that allow turn on of cholesterol-- biosynthesis and LDL biosynthesis, which then allows uptake of cholesterol from the diet?

OK, so that's the question. And what does this look like? And people hadn't measured this by any method, and this model I've gone through a number of times in class today, so I'm not

going to go through it again. Hopefully you all know that in some form in your head, or you have the picture in front of you so you can remember it.

So these are the questions I want to pose, and I want you guys to do the talking today. And what I'm going to do is, I have most of the figures on my PowerPoint, so we can bring them up and look at them. And you can tell me what you see. And then everybody might be seeing something different-- and so we're thinking about this differently, and maybe we come to some kind of consensus about whether these experiments were carried out well or not.

So one of the first things-- so these will be the general things, and then we'll step through them. But they wanted to perturb the cellular cholesterol levels. And how did they end up doing that?

Did that make sense? We talked a little bit about this already. I mean, what did they use as tools to do that?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: So you need to speak louder, because I really am deaf. Sorry.

AUDIENCE: So just, right here, they were careful of the amount of cholesterol present in this?

JOANNE STUBBE: So that's one place, so they can deplete cholesterol for the media. But then what did they do? So the whole paper is about this-- how did they control the [INAUDIBLE]? Let's assume that they can do that, and they got good at that. I think a lot of people have used that method, and so they can deplete media.

So how did they deplete cholesterol? There was some unusual ways to deplete cholesterol in this paper. Did any of you pick up on that?

AUDIENCE: A chemical that could bind to cholesterol.

JOANNE STUBBE: So did you think that was unusual? Did any of you look up what that was?

AUDIENCE: It was a kind of carbohydrate that can bind to cholesterol.

JOANNE STUBBE: Yeah, so but what was interesting about it, it was hydroxypropyl-- remember HP, cyclodextrin. We're going to look at this in a minute. But what do we know-- what was the other molecule they used to add cholesterol back?

AUDIENCE: Another form of that molecule is--

JOANNE STUBBE: So methyl-cyclodextrin-- I'm going to show you the structure, but they aren't very different. So have any of you ever heard of cyclodextrin before? People won the Nobel Prize for that, Don Cram won it, Breslow spent his whole life studying host guest interactions. So you guys, I don't know what you teach you now anymore, but that used to be something that was taught a lot, host guest interactions, trying to understand weak non-covalent interactions as the basis for understanding catalysis.

But to me, that was-- immediately when I saw this, what the heck's going on? So then I Googled it, and immediately-- and I don't know anything about hydroxypropyl-- you Google it, you look it up. And then you look at it, and if you were a chemist and you were really interested in the molecular interactions, you might make a model of it. And then see, what is the difference between that one little group, when you look at the structure, it's amazing.

And that's the basis of most of the experiments. So you need to believe that they figured that out. And that's not in this paper, so if you really cared about it you would have to go back and read earlier papers, and see what are the experiments that led them to focus on these molecules?

How else did they end up getting cholesterol levels back into the cell? Do you remember what the other method was? So we'll come back and we'll talk about this in a minute-- so that was one of the methods.

AUDIENCE: They added two kind of sterols.

JOANNE STUBBE: OK, so they did add two kind of sterols-- and they tried to figure out, this is another unknown, what was the difference between the sterols? Simply a hydroxyl group. OK, so if you looked at this, cholesterol is this guy.

And then they had something like this guy-- 25, and remember where [INAUDIBLE] the side chain, hanging out of the little [? cheer ?] system you have. I don't think they learned very much from that. And in fact, in your problem set, you had all of these different cholesterol analogs.

I mean, I think we still really don't get it. That's complicated-- we talked about this in class. You have these transmembrane helices-- what is it that's actually the signaling agent? So people

are still asking that question, and we haven't quite gotten that far. But if you've read the reading, for HMG CoA reductase degradation, which is what we we're going to be talking about in class, the signaler is not the sterile, it's lanosterol.

OK, and where have you seen lanosterol? The biosynthetic pathway has lanosterol sitting in the middle. It's not all that different, structurally, from cholesterol. You need to go back in, they all have four-membered rings, they have different extra methyl groups.

So people are trying to sort that out. I don't think we really know. But how well? So you're right, they use sterols. They didn't use that, they didn't see very much difference with the sterols. What was the other way, which is sort of unusual, that they added cholesterol back into the system. So they could add it back with the methyl cyclodextrin-- they told you that that worked, and if you believe that-- and you look at the data-- it looked like that was happening.

Nobody remembers? OK, well, we'll get to that in a little bit.

OK, so the question we're focusing on is what are the changes in concentrations of cholesterol in the ER? So what method did they use to try to separate the ER membranes from all the other membranes?

AUDIENCE: They first separated the [INAUDIBLE]--

JOANNE STUBBE: They separated the what?

AUDIENCE: The sterols and the nucleus in the [INAUDIBLE].

JOANNE STUBBE: OK, so that's good. You can separate out the nucleus, and you could do that by ultracentrifugation-- we've seen that used in different kinds of ultracentrifugation. We've seen the different particles, the lipoproteins in the diet, how do we separate those?

We talked about that in class briefly, you haven't had any papers to read. But what was the method of separation? If you look at all those particles-- remember we had a little cartoon of all the particles, and we focused on LDL, which is the particle that has the most cholesterol. So that's why everybody is focusing on that.

What was the basis of the separation?

AUDIENCE: Was it sucrose screening?

JOANNE STUBBE: Was the what?

AUDIENCE: Was it a sucrose screening-- the ultracentrifugation?

JOANNE STUBBE: You need to--

AUDIENCE: Did they use a sucrose screening, like ultracentrifugation?

JOANNE STUBBE: Yeah, ultracentrifugation. But how did the--

AUDIENCE: For the sucrose screening?

JOANNE STUBBE: Yeah, OK, so they have different density gradients. , OK so that's going to be a key thing, and that's because if you look at the composition, they have different amounts of proteins, different amounts of fats. And they have different-- they float differently. So that's the method that they're going to use here.

Is that a good method? Can you think of a better method? So in order to understand the switch for cholesterol, you've got to be able to measure the changes in cholesterol. Not an easy problem, because cholesterol is really insoluble in everything. And so how much is really in there, and how does it change under different sets of conditions?

So is this a good method? What do you think? We'll look at the method in a little more detail, when I pull up the figures, but what did you think when you read the paper?

AUDIENCE: Seems a pretty good method, other than that they're slightly different any other like properties different from the membrane than say, press on golgi bodies and ER. So it's like the only one I can think of.

JOANNE STUBBE: Yeah, so the question is, you could you separate? Even separating the nucleus from the cytosol is not so trivial. But these methods are really gross methods, and during the centrifugation, things diffuse. So if you're having close separations, it's a equlibrating down this thing. And so you're getting your proteins, or your lipids are spreading out.

Is there anything else any of you experience with insoluble-- this is what we're dealing with, is an insoluble mess, and how do you how do you separate things in a way that you have control over it so that you can address the key questions in this paper?

Nobody thought about anything else? Did you like this method? Were you convinced by the

data?

AUDIENCE: I mean, like I couldn't necessarily think of something better. I don't know, I guess the thing that sketches me out the most about it just like how-- I'm not really familiar with the method. I haven't done this myself, so I don't know how that process affects the membrane integrity.

JOANNE STUBBE: So that's an incredibly important question, because lipids confuse. They can mix. The question is, what are the rate constants for all of that? And we don't really teach very much in the introductory courses about lipids, and they're partitioning between other membranes and fusion, and all that stuff.

But if you think about it, that's what the cell is, right? How do you get a plasma membrane, and all these membranes around all these little organelles-- that's an amazing observation. And we've seen in class already, what have we seen to get LDL receptor from here to the plasma membrane? How do we have to do that? We had to use these little vesicles.

So you're generating something over here, it goes through the Golgi stack. Again, another set of membranes has got to come out the different levels of the Golgi stack. And then it's still got to get into the plasma membrane, and fuse, and dump its cargo. So I think it's an amazing process.

And people interested in evolution, this is one of the major things people are focused on is, how can you make cells, little fake cells, artificial cells, that can replicate themselves. You can make it, and they're going to have to divide and fuse. And it's exactly the same problem here.

And so this question of fluidity is an extremely important question. And a lot of people that focus on lipids-- which is not a popular thing to study, because it's so hard-- it's incredibly important. And people that look at membrane proteins, they almost always have lipids on them. And when you do them yourself, you have a detergent, which is not a real lipid-- does that change the property?

So all of these questions, I think, are really central to what happens in the membranes, which is a lot of stuff inside the cell. So I think it's good to question what they did. I think their results turned out to be quite interesting. But we'll come back-- I think that was a hard problem. And so we'll come back and we'll look at this.

And so then, let's say that we could end up separating things. Then the question is, what was the key type of measurement they made, where they could correlate the changes in

cholesterol levels-- we talked about, you can control perhaps the cholesterol levels with the cyclodextrin.

But then, how did they correlate the changes in the cholesterol levels in the membrane with this transcriptional regulation? Which, that is what happens with the steroid-responsive element-binding protein, the transcription factor. So what happens in that process? What are the changes in the SRE BP dependent on the concentrations of the cholesterol? And how did they take advantage of that in answering this question about what the cholesterol levels were that allowed you to turn on transcription of LDL receptor, and HMG CoA reductase.

So what's the major assay? We'll look at that, as well. So if you go back and you look at the model, what happens in this model? All right, here we go-- what happens in this model? What's happening to SREBP?

AUDIENCE: It has completely changed and exposed [INAUDIBLE].

JOANNE STUBBE: No, that's SCAP-- SCAP, that's this guy. OK? So SCAP, that's a key player. That's what we talked about. I know the names are all confusing. You're going to need to write these down to remember. The names are very confusing. Yeah?

AUDIENCE: So the SCAP SREBP, whatever you call it, complex move signal g-apperatus then part of it's cleaved and moves to the nucleus?

JOANNE STUBBE: Right, so how could you take advantage of that? This is the key observation that they're taking advantage of, to ask the question-- since this whole process is dependent on the concentration of cholesterol. If you have high cholesterol, there's no way you want this to happen-- you want to shut it off.

If you have low cholesterol, you want to turn these guys on. So this movement is the key. And what do we see, if we look at what happens to this protein, SREBP, what happens to it during this process? It gets cleaved. And how could you monitor that cleavage? How do they do it in the paper?

AUDIENCE: They used a-- was it a [? florifor-- ?] or is that the homework?

JOANNE STUBBE: They could use a [? florifor, ?] they didn't do that. They did a what?

AUDIENCE: They were able to separate the [INAUDIBLE] gel?

JOANNE STUBBE: So it can be operated by a gel. So to me, this is quite an easy assay. Because if you look at this-- I don't remember what the molecular weight is, but it's a lot smaller over here. And so, that turns out to be a great assay. So that part of their analysis, I think, was a really smart part of the analysis.

And so then the question becomes, can you quantitate all of this? So if you have a lot of cholesterol, this doesn't happen. And so everything is bigger, and resides in the membrane. You could even probably look at that. Whereas, when the cholesterol is really lower, things go there.

And it's everything in between. The question is, what is the concept-- can you measure if you have X% cholesterol in the ER, how much do you have to decrease it to see a change or a switch in where this protein goes? So I think the experimental design is actually amazingly creative. But then you see the data of the other side.

And what I want to do now is focus on what the issues are. So we're going to come back and look at, how did they look at SREBP? So you could look at this a number of ways-- you could look at this by protein gel directly. How else do people look at proteins using westerns? What's a western?

Anybody know what a western analysis is? Didn't I ask you that at the beginning of class? How else do you detect proteins? You've seen this in the first half of the semester a lot.

Yeah, antibodies. So if you have antibodies to this-- and we'll talk about this, because the western analysis, which people use all the time, and there are so many issues with it, that I think I want you to think about what the issues are.

And then you correlate the two-- changing the levels of cholesterol. Which they measure by mass spec after separation and purification of lipids, and the cleavage. And they plot the data, and that's where they got the analysis from.

So the first thing that you want to do-- the first thing, and the key to everything, is separation of the membranes. And so, this is a cartoon of when you put something, you load something on the top, and you have a gradient, and the gradient could be made of a number of things.

Have any of you ever run these kinds of gradients? OK, so you can make them out of glycerol,

you can make them out of sucrose-- did anybody look at how these gradients were made? Did you read the experimental carefully enough to look at that?

Yeah, how do you make a sucrose gradient? You have no idea? But yeah, so layering.

So what you really like to do is have a continuous gradient, or something. But sucrose is incredibly viscous. So if you were trying to make a linear gradient, which you could do by mixing two things of different concentrations-- if you could get them to stir really well, and then add it in, and you could generate a gradient. But it's so hard to do, that what happens is they end up layering it.

So they make X%, Y%, Z%, they put it down. And then they try to layer something on top of it. And then they put whatever the interest in at the top, and then they centrifuge it.

So what are the issues? Do you think this is what the gradient would look like? So what are the issues when you're doing this, when you layer it?

And this is why the data-- which we'll talk about in a minute-- is the data, or part of the issue is this method. That's why you need to think about the method. And there are better ways to do this. And it really depends on what you're trying to separate.

So if this band-- say these were two bands, you wouldn't really get very much separation at all. If there were two separate things that sedimented under these conditions very close together.

So what would happen when you're sedimenting this? Does anybody have any idea how long it takes? Do you think you'd do this in a centrifuge, you spin it for three minutes, and then-- so sometimes you sediment these things for 16, 20 hours. So what happens during the sedimentation? That might make this more challenging, in terms of separating what you want to separate?

AUDIENCE: I'm not sure, but it [INAUDIBLE] diffusion.

JOANNE STUBBE: Yeah, so exactly, you have diffusion. And even when you've layered things on top of each other like that, you start to have diffusion. And if you shake up the tube a little bit, it's all over. So how do you prepare these things is not-- so people still use these methods, but I would like to see better methods.

And so they tried one method with sucrose, and then that wasn't good enough. We'll look at

the data. So they went to a second method. And where did they come up with this?

I have no idea where they came up with this, but there was an MD PhD student in our class who had seen this and one of his classes, and they use it and some blood test. So I think that's probably where these guys got it from, because Brown and Goldstein are both MDs. But again, it's just another way to make a gradient.

And I'm not sure why this gradient works as effectively as it does. But the first gradient didn't work so great, and we'll look at that data. So then they added on a few more steps, because they weren't happy with the level of separation.

So looking at membranes, I think this is going to be more and more looking at membranes, because membranes, you have two leaflets-- the lipids and the leaflets are different. Do you think that affects the biology? I guarantee you it affects the biology in ways that we would really like to understand that I don't think we understand very well.

When you isolate a membrane protein, have any of you ever isolated a membrane protein? So you have an insoluble-- it's in this lipid system. How do you think you get it out, so you can go through the steps, a protein purification that you've talked about, or you have probably done in an introductory lab course? What is the first thing you need to do?

Yeah, solubalize it. And how do you solubalize it?

AUDIENCE: With a detergent.

JOANNE STUBBE: Yeah, with some kind of detergent. It's like what you saw with a kilo microns, or the bile acids that we talked about. So you can use different-- and people have their own favorite detergents. But again, that changes things. But otherwise, you can't purify anything unless you happen to have a membrane where the only protein in the membrane is the one you're interested in, which, of course, doesn't exist.

So anyhow, they went through that. And then what did they end up seeing? So they went through different steps, and they separate them into different-- the supernate, or the light and the heavy membrane fractions. And then they have to analyze it. And so the question is, how do they analyze to tell how well these separations actually worked? What was the method that they did to determine whether they separated the ER from the plasma membrane, from the Golgi stacks, from the lisosomes, from the peroxisomes.

So they have all we have all these little organelles in there. What did they do to test each one of these fractions?

Let me ask you this question-- how do you think they got the-- how do you how did they get the material out of these gradients to do the experiments that I was just talking about. So they want to analyze what's in each of these bands. How did they get it out of this tube?

AUDIENCE: Would they use a Pasteur filter?

JOANNE STUBBE: So what do you think? You just stick it down in and suck it out? Well, I mean, yes, so what do you think? You could do that-- you open the top, you stick it in, you carefully stick it in. If you can see it. Lots of times you can see these lipids, because they're opaque, or something. So you can see.

Or, if you still hope your sucrose layers, lots of times they layer in between the different concentrations of the sucrose, and you see white stuff precipitating. So you could conceivably stick a pipe head from the top and suck it out.

AUDIENCE: But that would perturb all the other layers.

JOANNE STUBBE: Absolutely it would perturb all the other layers. So here you're doing something-- it's already a very hard experiment, because they're all being perturbed anyhow, because of diffusion. So is there any other way you could think about separating these things? And so, the hint is that they use plastic tubes.

So these things are not glass. Most centrifuges--

AUDIENCE: Freeze it? Cut it?

JOANNE STUBBE: Well, so you don't do that, that could be-- OK, so you could. But you then have to, if you were cutting it, you still have to get it out of the tube. Unless you had a saw that didn't have any vibrations when you were cutting it, of course, which would not happen.

But if you look here in this cartoon, so I gave you this, what are they doing here? They're sticking a syringe in through the side of the tube. And that's still what people use. So you can suck out-- if you can see something. So you have to be able to see in some way to know where to suck it out, so you might have a way, actually, in doing ultracentrifugations.

I think with the lipids you can see them by eyeball, but you might look at absorption. If they

have proteins, you could monitor absorption through the gradient, and that might tell you how to fractionate things.

But anyhow, that's also an issue. Because before they can do the next step in the analysis, they've got to get the material out.

So they've got the material out in each of these steps, and then, how do they look at this? They can pull it out. So what are they looking for? To tell them how effective this method is.

AUDIENCE: Maybe some specific markers for each protein.

JOANNE STUBBE: Exactly. So what are they-- to do that, what they're going to have to do is, before we look at the details of the method, I want to go through a western blot. So what do we know about a western blot?

AUDIENCE: I have a quick question about the method here.

JOANNE STUBBE: About the which method?

AUDIENCE: The lysis method [INAUDIBLE] ball bearing homogenizer. So they're literally putting these cells in something like a bunch of ball bearings?

JOANNE STUBBE: Yeah, you could do that. There's a lot of ways to crack open cells. I don't know which one's the best-- mammalian cells are really easy to open. Sometimes what I like to do is freeze and thaw them-- sometimes you have like a little mortar and pestle, or something like that. But that's-- I mean, yeast cells, you roll them.

You have to have enough cells so you can do something. If you only have a tiny amount of cells, it makes it really challenging with beads, because it covers the beads.

AUDIENCE: Do you have any issues with any of the different types of membranes that--

JOANNE STUBBE: Sticking to that? Absolutely. I'm sure you have to look at all of that kind of stuff. So how you choose, that's an important thing to look at, how you choose to crack open the cells. And it's the same with bacterial cells-- there are three or four ways to crack open the cells. And I can tell you only one of them really works efficiently.

And a lot of people, when they use some of the others, they do something and they assume it works, but they never check to see whether the cell walls have been cracked open. A lot of

times they haven't, and so what you get out is very, very low levels of protein, because you haven't cracked open the cell.

So figuring out-- mammalian cells are apparently, I haven't worked with those myself, but they're apparently much easier to disrupt than bacteria. Or if you look at fungi-- fungi are really hard to crack open, yeast. So anyhow, that's an important thing to look at.

So every one of these things, again, the devil is in the details. But when you're doing your own research, it doesn't matter what method you're looking at. The first time around, you need to look at it in detail, and convince yourself that this is a good way to chase this down. And you look at it in detail the first time around.

And when you convince yourself it's working really well, and doing what you want to do, then you just use it. And that's the end of it. You don't have to go back and keep thinking about this over and over again.

So the method we're going to use is a western blot. So we've got this stuff out, and have you all run SDS page shells? OK, so SDS page shells separate proteins how?

AUDIENCE: Based on size...

JOANNE STUBBE: By the what?

AUDIENCE: It separates into a a charge gradient, and then-- not a charge gradient, but--

JOANNE STUBBE: Not charge.

AUDIENCE: That's what drives the protein, but...

JOANNE STUBBE: Right, but it's based on size, because it's coded-- every protein ratio is coded with this detergent, sodium dodecyl sulfate, which makes them migrate pretty much like the molecular weight. But if you've done these, it's not exactly like the molecular weight. You can do standards where you know the molecular weight, you can do a standard curve, and then you see where your protein migrates. And sometimes they migrate a little faster, sometimes a little slower, but it's OK.

So you run this, and then what do you do? Does anybody know what you do next, to do a western?

AUDIENCE: You need to use the membrane to...

JOANNE STUBBE: Right, so the next thing they did was they used-- I'm going to put all of these up-- so they transferred it to a membrane. And why did they have to transfer it to a membrane to do this analysis? This is an extra step. And it turns out-- we're going to look at an antibody interacting with a protein. Why don't we just look at the antibody interacting with the protein to start with?

AUDIENCE: It doesn't have access to the protein.

JOANNE STUBBE: Right, it doesn't have very good access. It's really not very efficient.

So people found, pretty much by trial and error, that you needed to transfer this to a membrane. I mean, we have hundreds of kinds of membranes. How did they choose nitrocellulose? If any of you have one run westerns, you remember what kind of a membrane you used?

Did you use nitrocellulose? You do this in undergraduate class, don't you? You don't do a western? We used to do--

AUDIENCE: Did it once in undergrad class.

JOANNE STUBBE: Yeah, in what kind of a membrane? Was it in biology?

AUDIENCE: Yes, biology.

JOANNE STUBBE: So what membrane? Do you remember what the membrane was?

AUDIENCE: I think it was-- it was not nitrocellulose.

JOANNE STUBBE: It's not nitrocellulose. So this PVDF, polyvinyl difluoride is the standard one that people use now. It works much better than nitrocellulose-- this paper is really old, and so they're looking at nitrocellulose. So then they do this. And then, what do they do next?

They have an antibody-- we'll look at the details of this in a minute-- that can recognize the protein, that can find it on the membrane. And then what we're going to see is-- you still can't see anything really, because you don't have very much material there. And you can't observe-- you don't have enough to stain, oftentimes, by Coomassie, so you're going to have to amplify the signal.

So then you're going to make an antibody to an antibody. And then you have to figure out how

to, then, amplify the signal. And we'll look at that in a second.

Is this what-- you ran a western, is this what westerns look like?

AUDIENCE: I remember, we first [INAUDIBLE] non-specific proteins to occupy the sites.

JOANNE STUBBE: Yeah, so that's good, you have to block everything, if you're using crude extract. So in this case, we would be using the crude mixture-- well, not a crude mixture, it's been fractured by the ultracentrifugation that's been fractionated. But you still have mixtures of proteins in there.

Have any of you ever looked at westerns in a paper? Or even the papers you had to read? The paper on the PC-- go look at the PCK-- PCSK9 paper, that had westerns in it. What do you see? Do people show you something that looks like this? And if they did show you that, what would it look like?

So you have an antibody that's specific for the protein of interest, whatever that is-- supposedly specific. What do you see? What do you think you see? Do you think antibodies are specific?

I think I have an example of a typical western.

AUDIENCE: I don't think they're as specific as [INAUDIBLE]

JOANNE STUBBE: Yeah. Yeah. So when you look at a paper, you should pay attention to this when you read a paper, if you're doing anything in biology, what do you see? You never see a gel, ever. What you see is a slice of a gel where they cut off this-- the way they cut up all this stuff and all this stuff. The reason they do that is because it's a hell of a mess.

So let me just show you a typical-- I don't care what kind of an antibody you're using, in crude extracts, it's a mess. Because you have non-specific interactions. We'll just look at that.

So that would be something like you might see-- depending on how much antibody you have. So when you see this, the reason everybody reports data like that now. So it looks like it's really clean, but in reality-- I think if it is dirty as that, then in my opinion, I would make you publish the whole gel.

But people don't do that. They just cut off the little band they're interested in-- they can see it change in concentration using this method. But you should be aware of the fact that antibodies in general aren't as specific as you think they're going to be. Yeah?

AUDIENCE: Are they required to report the whole gel in supplementals?

JOANNE STUBBE: I mean, I think, it probably depends on the journal, and it probably depends on the reviewer. But I would say, we're going away from data-- is something that is a pet peeve for me. And all the data, which I think is all right, is published in supplementary information, as opposed to the paper.

I think if you have something really dirty, you should publish in the paper, in the main body of the paper. If you have something that's really clean, and it looks like that, it's fine with me. You don't even have to publish it, if you could believe what people were saying. Because people know what this looks like, a lot of people-- everybody uses westerns.

But if it's a real mess, then you need to let your reader know that this is not such an easy experiment, and it's not so clear-cut. That's what your objective is, is to show people the data from which you drew your conclusions. And then they can draw their own conclusions, which may be different.

So let's look at the apparatus to do this. So how do you get from here to here? So you have a gel, you run the gel, a polyacrylamide gel-- what do you do?

AUDIENCE: Put the membrane on the gel.

JOANNE STUBBE: So you put the membrane on the gel. And what do you do?

AUDIENCE: [INAUDIBLE] applying charges to.

JOANNE STUBBE: Yeah, so you're transferring it based on applying a voltage across this system. So here's your gel. And here's your membrane, nitrocellulose membrane. And then they have filter paper above the gel, and below the membrane.

Why do you think they have the filter paper there? When you ran the gel, did you have filter paper?

AUDIENCE: Yes.

JOANNE STUBBE: Yeah. How do you think they decide how to do this transfer? Do you think is a straightforward? Do you run it for an hour, do you run it for five hours, do you run it for 15 minutes? What is the

voltage you use to do the transfer? Do you think any of that is hard to figure out?

So how do you figure that out? Somebody told you that this is a good way to do it? Yeah, so that might be a place you start.

So you do it because somebody gave you a recipe. But then what do you need to do to make sure this recipe is correct?

AUDIENCE: Find out what conditions that work for what you're working on.

JOANNE STUBBE: Right, and then how do you do that? So that's true, every protein is going to be different. And if you have a protein-- if you have a clean protein, versus a mess of proteins, and you try to do this transfer, the transfer conditions will be different. So for example, if you really want to look at the concentration of something inside the cell, in the crude extracts, you never compare it to a standard with clean protein, because this transfer is different.

So you need-- in the back of your mind, if you care about quantitating this, you need to understand the basis of the transfer. So why do you think they have these filter papers here? So this goes back to what controls you would do to see whether your transfer was working. So what would you look for?

Did you do this? What did you do? What did you do with the filter papers in your--

AUDIENCE: You want to filter all to the SDS molecules...

JOANNE STUBBE: You did what?

AUDIENCE: You want to filter all--

JOANNE STUBBE: No, that's not what you do. I mean, you might want to do some of that, too, but in terms of thinking about whether your transfer is successful-- figuring out the conditions to blot from the gel to a piece of paper is not trivial.

And there is a standard way that you do this, initially, to try. But then you have to make sure that that method is working. And lots of times it doesn't work. So it's something that's going to be experimentally determined.

So the question is, what would you think would happen if you did this for six or seven hours? Whereas, a normal blot would take two hours?

AUDIENCE: Would be transferred onto the filter paper?

JOANNE STUBBE: Right, it would go right into the filter paper, or even off the filter paper. So what you do is you take the filter paper out, you look for protein being bound. What about the gel? What do you do with the gel after your experiment's over?

AUDIENCE: Make sure a protein's not on it?

JOANNE STUBBE: Right, make sure that the protein is not on it. So these are simple controls, but these are the controls you always do until you work out the conditions to make sure this works. And it's pretty critical to make sure you have good transfer.

So then, so this is the antibody thing that they do. Has anybody thought about these kinds of assays? You've seen them, I think, already in class. But what's wrong with this picture? The target protein, what's wrong with this picture in the target?

So here's your nitrocellulose filter paper. What's wrong with this cartoon? Should be unfolded, yeah. So you're doing SDS page, it's unfolded.

So then we react it with an antibody. Presumably we have a good antibody, but you've already learned in the first half of this course that having really good antibodies is not so trivial-- you can get them, but most of the time they are not specific if you're looking at crude extracts. They have little epitopes they recognize, if you're using monoclonals that could be present in other proteins.

And furthermore, how are you detecting something? An antibody as a protein, it has absorption of 280. Again, this is too low to see, so putting an antibody on it is still going to be too low to detect. So how do you detect your signal?

So have you done this? I'm surprised they don't do this in your introductory class-- they don't do westerns, at all.

So what you're looking at is an antibody to an antibody. So you put your antibody on, that's specific for your protein. And then you make an antibody in another organism that can specifically recognize antibodies in general. So if this is to a mouse, you make it to go and isolate that.

And then what you do is derivatize the second antibody with what? A protein? That can

function as a catalyst.

AUDIENCE: Why can't you just derivatize the first antibody?

JOANNE STUBBE: Well, what? What did you say?

AUDIENCE: It's more expensive?

JOANNE STUBBE: Well, no, I don't know whether it's more expensive or not. But--

AUDIENCE: Well, because you'd have to derivatize every primary antibody.

JOANNE STUBBE: So you'd have the derivatize every primary antibody, and so this is a standard procedure. You could derivatize the primary antibody. So that's not a bad question.

And so what you're doing now, you can buy these commercially, so they have rabbit, rabbit, mouse, whatever, antibodies. And the key is the amplification of the signal, and you use enzymes to amplify the signal. Does anybody know what the enzymes are, what the enzymes do to amplify the signal?

AUDIENCE: You can covert the molecule to a blue molecule...

JOANNE STUBBE: To something that's colored. So does anybody know what that horseradish peroxidase-- have you ever heard of horseradish peroxidase? So that's a heme iron-- we're going to be talking about heme irons pretty soon, and hydrogen peroxide. It makes a chemically very reactive iron oxide species, that can oxidize a dye that changes color. And it has extremely high extinction coefficients.

So you can see it, and it does it catalytically and the lifetime of the dye is long enough. So it accumulates, and you can get really amplification of your signal. Or you can use a phosphatase that liberates something that's highly colored, again, and you can see it.

So this is a standard method that everybody uses. And so, that's our gel. So now we're looking at sort of-- at the end already-- but we're looking at these gels, and what do you see through the different steps? So if we look through the first gradient, through the sucrose gradient, that gets us through DNE.

And if you look, say, at lane E-- our goal is to separate proteins that are specifically localized in each one of these membranes. So you need to believe that's true, that people have selected

the right group of proteins to look for. And you notice they do more than one. So they look at multiple proteins.

Why do you think-- do you think it's easy to select the proteins to look for? And why or why not?

So, they obviously have selected a group of proteins, and I think most people would agree that they've selected a good group of proteins. But what do we know now about proteins, do they stay in one place? No, they move around. But some might be present in very low amounts, sometimes in much higher amounts.

And so you need to have more than one protein as a control to make sure you're looking in the right region. And what do you see in E? If you look over here, it tells you what the organelle is. And if you look at this protein, this is localized to the lisosomes-- we talked about that in class. If you looked at this protein, it's localized to the peroxisomes.

So in addition to the ones we care about, the ER proteins, we're also getting proteins that are localized in other membranes. So that's when they went to the next method, and they added on another gradient to try to separate out, again, the lysosomal and the peroxisomal proteins.

And you can see they were pretty successful at this. There's none of these proteins left in this gradient. So that's good. And they took it a step further. Do you remember what this is? What are they looking for down here, in this?

AUDIENCE: Enzymatic activity.

JOANNE STUBBE: Yeah, so enzymatic activity is localized in certain organelles. So they again did a second experiment to look at all of that.

So they were very careful in this, they figured out how to separate. And that's the key thing for them to analyzing the concentration of cholesterol in these membranes. And what they looked at-- we're over time-- but is the concentration of cholesterol compared to the total amount of lipids.

And how did they do that analysis? Gene Kennedy, who's at Harvard Medical School-- he's in his 90s, now-- really trained all the lipid chemists in the whole country. And they figured out many years ago how to separate lipid fractions with methanol, chloroform extract, something that you guys probably haven't though about at all.

But we're really pretty good at separating things, and it's nothing more than an extraction like you do as organic chemist to purify and separate things. We've figured that out.

And so then they use mass spec to allow them to quantitate the amount of glycerol. And then in the end, so they use mass spec, these western blots, and they can change the concentration of the cholesterol and do the experiments over and over again, to see what happens. And when they do that, this is the picture of cyclodextrin.

So you can see the only difference is this group here versus that with a methyl. And one, so this is hydroxypropryl-- hydroxypropionyl cyclodextrin-- so it's like a cavity like this. And the other only other change here is a methyl group, removing that.

And they have very different properties about binding and releasing cholesterol, which somebody had to do a lot of studying on to be able to ensure that they can use it to remove cholesterol, and then to add it back to the media. And so you have to think about the exchange kinetics, you have to think about a lot of things. This is not trivial to set this up, to figure out how to control the levels of cholesterol.

And then what they do is, this is like a typical assay, and this is the end. What you can do is this, removes cholesterol, and you can see it change. This reports on low levels of cholesterol, which is happening over here, allows the protein to move to the nucleus where it's smaller. And that's how they do the correlation-- the correlation between the levels in the nucleus and the levels of cholesterol.

So I thought this was a pretty cool paper. And these kinds of methods, I think, will be applicable to a wide range of things if people ever do biochemistry, looking at the function of membranes.

So, OK, guys.